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I, KIM MARSHALL , MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed are true copies of the Provisional specification in connection with Application No. PO 6962 for a patent by MACQUARIE RESEARCH LTD, AUSTRALIAN WATER TECHNOLOGIES PTY LTD and BECTON DICKINSON AND COMPANY filed on 19 May 1997.

I further certify that the annexed specification is not, as yet, open to public inspection.

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Patents Act 1990

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PROVISIONAL SPECIFICATION

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Invention Title:

Antibodies to Cryptosporidium

The invention is described in the following statement:

Technical Field

The present invention relates to antibodies to *Cryptosporidium* and methods to raise suitable *Cryptosporidium*-specific antibodies in animals.

Background Art

The protozoan parasite *Cryptosporidium* is amongst the most common pathogens responsible for diarrhoeal disease in humans. Infection occurs when *Cryptosporidium* oocysts shed in the faeces of infected individuals are ingested by new hosts. Recently, several large outbreaks of cryptosporidiosis have occurred in which drinking water has been identified as the source of infection. Surveys have shown that many surface water supplies are contaminated with *Cryptosporidium* oocysts.

Laboratory methods used to detect *Cryptosporidium* often involve the use of antibodies to this organism. Typical methods used to analyse water samples for the presence of this organism include microscopy and cytometry or a combination of these techniques. Flow cytometric methods involve staining of samples with a fluorescently labelled monoclonal antibody specific to the surface of *Cryptosporidium* oocysts and then analysis with a sorter flow cytometer. Particles with the fluorescence and light scatter characteristics of *Cryptosporidium* oocysts are sorted onto a microscope slide and examined manually using epifluorescence microscopy to confirm their identity as oocysts. This confirmation step is necessary because with a single antibody the cytometer is unable to distinguish oocysts from all other particles present in water samples. The particles that the cytometer can mistake as oocysts are autofluorescent particles such as algae or particles that non-specifically bind the oocyst-specific antibody.

Analysis-only flow cytometers are available which are simple to operate and relatively inexpensive. These cytometers are unable to perform sorting. To enable the detection of *Cryptosporidium* oocysts using an analysis-only cytometer the discrimination achieved by the cytometer must be improved so that non-oocyst particles are not mistaken as oocysts. The present inventors have shown previously that it is possible to detect a single specific microorganism in turbid water samples with an analysis cytometer if the microorganism is labelled with two different antibodies.

Unfortunately, the antibodies for *Cryptosporidium* presently available are not ideal due their stickiness and there is a need for more specific and reactive antibodies to the surface of *Cryptosporidium* oocysts. Monoclonal

antibodies (mAbs) that are specific to the surface of *Cryptosporidium* oocysts are used for detecting *Cryptosporidium* in clinical and environmental samples. All available mAbs that bind to the surface of *Cryptosporidium* oocysts are of the immunoglobulin M (IgM) or IgG3 subclass. Monoclonal antibodies of the IgG1 or IgG2 subclass would be preferable because they usually show less non-specific binding. Such mAbs would be more suitable in methods currently used for the detection and identification of *Cryptosporidium*. Unfortunately, past attempts by workers in the field to produce IgG1 or IgG2 monoclonal antibodies to *Cryptosporidium* have been unsuccessful or not substantiated (Smith, 1994; MacDonald *et al.*, 1991). It is generally considered that due to the antigenic characteristics of this organism, this class of antibody is not produced by infected or immunised animals (Smith, 1994).

In a previous Australian provisional patent application of the present inventors (PN2831), monoclonal antibodies to a range of *Cryptosporidium* oocyst antigens were developed. Whole or excysted oocysts that were exposed to various treatments were used as antigens. From a total of 8 fusions that included screening several thousand hybridomas only one hybridoma was identified that was specific to the surface of *Cryptosporidium* oocysts. This monoclonal antibody was of the IgM immunological subclass.

The present inventors have now developed a new method that allows the production of IgG1 or IgG2 antibodies to the surface of *Cryptosporidium* oocysts.

Disclosure of Invention

In a first aspect, the present invention consists in a method to produce IgG1 or IgG2 subclass antibodies reactive to the surface of *Cryptosporidium* oocysts comprising:

(a) pretreating *Cryptosporidium* oocysts with a reagent so as to remove the surface layer of the oocysts to form an oocyst antigen preparation;
(b) separating the oocysts from the antigen preparation so as to obtain an antigen preparation capable of eliciting a detectable IgG1 or IgG2 immune response in an animal to the surface of the oocyst;
(c) immunising an animal with the antigen preparation so as to elicit an IgG1 or IgG2 immune response in the animal; and
(d) obtaining IgG1 or IgG2 antibodies reactive to the surface of *Cryptosporidium* oocysts from the animal.

It will be appreciated that once a suitable immune response has been stimulated in an animal, for example in a laboratory mouse, monoclonal antibodies of IgG1 or IgG2 subclass may be generated by standard techniques from that animal.

In a preferred embodiment of the first aspect of the present invention, the reagent used to prepare the antigen preparation is a detergent, preferably the detergent is sodium dodecyl sulphate (SDS). One suitable pretreatment involves boiling the oocysts in the presence of SDS for a sufficient time to generate a suitable antigen preparation. When a concentration of 0.5% (w/v) SDS is used, boiling for 1 hour has been found to be particularly suitable.

Other suitable reagents include urea, detergents such as Triton X-100 or nonident, enzymes such chitinase, oxidising agents such as sodium hypochlorite, sodium periodate, ozone and reducing agents such as mercaptol ethanol and 1,1,1-trichloro- 2,2-bis[4-chlorophenyl]ethane (DDT).

The pretreatment removes antigens from the surface of the oocyst in a form that will allow the generation of IgG1 or IgG2 antibodies when injected into an animal.

The animal may be immunised by any technique suitable for eliciting an immune response in an animal. Adjuvants may also be included with the antigen preparation prior to immunising the animal to promote a strong immune response in the animal.

In a further preferred embodiment, the antigen preparation also enhances the production of IgM antibodies when placed in an animal.

In a second aspect, the present invention consists in substantially isolated IgG1 or IgG2 antibodies reactive to the surface of *Cryptosporidium* oocysts produced by the method according to the first aspect of the present invention.

Preferably, the antibodies are monoclonal antibodies.

In a further preferred embodiment, the IgG1 monoclonal antibody is produced by clone CRY104.

In a third aspect, the present invention consists in the hybridoma clone CRY104.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples and the accompanying drawing.

Brief Description of Drawings

Figure 1 shows the comparison of monoclonal antibodies for staining oocysts in water samples.

Modes for Carrying Out the Invention

MATERIALS AND METHODS

***Cryptosporidium* oocysts**

Cryptosporidium parvum oocysts were purified from pooled faeces of naturally infected neonatal calves in Sydney. Faecal samples were centrifuged (2000 g, 10 min) and resuspended in water twice and then resuspended in 5 volumes of 1% (w/v) NaHCO3. Fatty substances were then extracted twice with 1 volume of ether, followed by centrifugation (2000 g for 10 min). Pellets were resuspended in water and filtered through a layer of pre-wetted non-adsorbent cotton wool. The eluate was then overlaid onto 10 volumes of 55% (w/v) sucrose solution and centrifuged (2000 g for 20 min). Oocysts were collected from the sucrose interface and the sucrose flotation step repeated until no visible contaminating material could be detected. Purified oocysts were surface sterilised with ice cold 70% (v/v) ethanol for 30 min, washed once in phosphate buffered saline (PBS; Oxoid, Sydney) and stored in PBS at 4^0C for up to 2 weeks.

Antigen preparation

Surface extraction:

A 2 ml sample of oocysts containing approximately 2×10^9 oocysts was centrifuged at 13000 g for 1 minute and the supernatant removed and discarded. Oocysts were resuspended in 2 ml of ice cold 0.5% (w/v) SDS and placed in a boiling water bath for one hour. The sample was then centrifuged at 13000 g for 20 minutes to remove the oocysts. The supernatant was carefully removed, mixed with 10 ml of acetone and placed at -20°C for 8 hours. The sample was then centrifuged at 13000 g for 10 minutes and the supernatant discarded. A small white precipitate was then resuspended in sterile PBS.

The protein concentration was measured using the commercially available Biorad DC protein assay using the standard protocol and bovine serum albumin (BSA) as a standard.

Oocyst walls:

Cryptosporidium oocysts were excysted as described by Robertson *et al*. (1993). A 1 ml sample of oocysts containing approximately 1×10^9

oocysts was centrifuged at 13000 g for 1 minute and the supernatant removed and discarded. Oocysts were resuspended in 1 ml of acidified Hank's balanced salt solution (HBSS), pH 2.7, and incubated at 37^0C for 30 min. Samples were then washed and resuspended in 1 ml of HBSS with 100 µl of 1% (w/v) sodium deoxycholate in Hank's minimal essential medium (HMEM) and 100 µl of 2.2% (w/v) $NaHCO_3$ in HBSS, and incubated at 37°C for 4 h. The sample was then analysed using the Coulter Elite flow cytometer as described previously (Vesey *et al.*, 1997). The population with the lowest forward angle light scatter signal was sorted into test tubes and concentrated by centrifuging at 3000 g for 20 minutes. Concentrated samples were stored at -20°C in PBS.

Immunisation of mice

Five Balb/C female mice were immunised by IP injection with either 200 µg of the oocyst surface extract (group E mice) or 4 x 10^4 oocyst walls (group W mice). Antigen preparations were emulsified in Freunds complete adjuvant. Mice were bled prior to receiving the primary injection to provide a negative control. Two further IP injections with the same amount of antigen but emulsified in Freunds incomplete adjuvant were given at 3 week intervals. Mice were bled after the second of these injections to check for immune response. Group E mice were given two final intravenous boosts of 200 µg of antigen were given 3 days and 1 day prior to the fusion.

Analysis of mouse serum

Samples (approximately 50 µl) of blood was collected by tail bleeding and then centrifuged at 13000 g for 30 seconds. The top layer of serum carefully removed and stored at -20°C until analysed. Serum was diluted to 1 in 100, 1 in 1000 and 1 in 10,000, in 1% (w/v) bovine serum albumin in PBS (BSA-PBS). Aliquots (50 µl) of diluted serum were mixed with 10 µl of oocyst suspension in PBS (containing approximately 1 x 10^6 oocysts) and incubated at room temperature for 20 minutes. Samples were mixed with either 50 µl of goat anti-mouse IgM specific antibody conjugated with FITC (diluted 1 in 50 with BSA-PBS)(Sigma product number) or with 50 µl of goat anti-mouse IgG specific antibody conjugated with PE (diluted 1 in 200 with BSA-PBS)(Sigma). After a further 20 minutes incubation at room temperature samples were analysed using a FACScan flow cytometer. A negative control of PBS and an IgM positive control of tissue culture supernatant from a IgM monoclonal antibody specific to the surface of

Cryptosporidium oocysts were analysed with each batch of samples. The mean fluorescence intensities of the FITC and the PE stained samples were recorded.

Samples of mouse serum diluted 1 in 500 in BSA-PBS were analysed using western blotting.

Production of hybridomas

Mice were sacrificed, spleen cells dissected and fused with NS1 mouse myeloma cells and the resulting hybridomas cloned. A 50 µl volume of tissue culture supernatant from each hybridoma was mixed with 10 µl of oocyst suspension in PBS (containing approximately 1 x 10^6 oocysts). Samples were incubated at room temperature for 20 minutes and then mixed with 50 µl of goat anti-mouse antibody specific to both IgM and IgG antibodies and conjugated with FITC (diluted 1 in 100 with BSA-PBS)(Selinus, Melbourne). After a further 20 minutes incubation at room temperature samples were analysed using a FACScan flow cytometer. A negative control of tissue culture supernatant from a *Disctyostelium*-specific antibody (MUD62) and a positive control of tissue culture supernatant from a IgM monoclonal antibody specific to the surface of *Cryptosporidium* oocysts (CRY26) were analysed with each batch of samples. Hybridomas that produced a higher mean FL1 than the negative control were cloned and tested once more.

The immunological subclass of monoclonal antibody produced by clones was identified using the Sigma Immuno Type Kit.

Flow cytometry

A FACScan flow cytometer was used for analysis of mouse serum and hybridomas. Logarithmic signals were used for all detectors. The threshold was set on side scatter at a value of 500. The detectors were set at the following levels of sensitivity: 200 for side scatter (SSC); E00 for forward scatter (FALS); 600 for the green fluorescence detector (FL1) and 600 for the red fluorescence detector (FL2). A region (R1) was defined on a dot plot of FALS versus SSC that enclosed single oocysts but not clumps of oocysts. Histograms of FL1 and FL2 were gated so that the only particles that appeared in region R1 would appear on the histograms. The mean value of FL1 and or FL2 from the histograms were recorded for 2000 oocysts from each sample analysed.

Western Blotting

Aliquots (100 µl) of oocyst suspension in PBS containing approximately $5x10^7$ oocysts were mixed with 100 µl of reducing sample buffer (0.12 M Tris HCl, 10% (v/v) glycerol, 3% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.02% (w/v) bromophenol blue) and boiled for 2 minutes. The entire sample was loaded onto a 10% SDS slab gel and run at 150 V for 1 hour. A sample of prestained molecular weight markers (Novex) was included. Following SDS-PAGE, the proteins were transferred to nitrocellulose using a semi-dry electroblotting system and a discontinuous buffer system. The nitrocellulose was then cut into 2 mm wide strips and soaked in 2% (w/v) skimmed milk in PBS. The strips were then incubated with either mouse serum or hybridoma culture supernatant. Tissue culture supernatant from an IgM *Cryptosporidium*-specific monoclonal antibody was included as a positive control. Samples were incubated for 1 hour at room temperature. The nitrocellulose strips were then rinsed for 3 x 5 min in 3% (w/v) skimmed milk powder in PBS; then incubated for 1 h in a goat anti-mouse antibody (specific to both IgM and IgG antibodies) conjugated to horse radish peroxidase (HRP, Tago, Inc. Burlingame, California, USA), diluted to 1:1500 with 3% (w/v) skimmed milk powder in PBS. Strips were then washed for 3 x 5 min in PBS and developed using a fresh solution of 4CN substrate and finally washed extensively under running tap water for at least 20 min.

Water samples

Samples (10 l) of untreated surface water were collected from locations around Australia and concentrated using a flocculation technique (Vesey *et al.* 1993a). A composite untreated surface water sample was prepared by mixing aliquots of samples from 15 different sites. The sample was centrifuged at 3000 *g* for 10 min and the pellet resuspended in PBS. Aliquots (50 µl) of water sample concentrate were seeded with 10 µl of oocyst suspension (containing approximately 1000 oocysts) and mixed thoroughly. The samples were then mixed with 50 µl of tissue culture supernatant from a *Cryptosporidium*-specific monoclonal antibody and incubated for 20 minutes at room temperature. An aliquot (50 µl) of a FITC conjugated goat anti-mouse antibody (specific to both IgG and IgM antibodies)(Silenus) diluted 1 in 300 in PBS-BSA was then added to each sample and incubated for 20

minutes at room temperature. Samples were then analysed using flow cytometry.

RESULTS

Analysis of mouse serum

Flow cytometric analysis of mouse serum revealed a large immunological response to the surface of *Cryptosporidium* oocysts in group E mice (Table 1). Twenty-one days after the second immunisation both IgM and IgG *Cryptosporidium*-specific antibodies were detected in the serum of group E mice. Oocysts stained with serum from group E mice fluoresced very brightly even when the serum was diluted to 1 in 10,000 (Table 1). The fluorescence of oocysts stained with serum from group W mice before and 21 days after the second immunisation were not significantly ($p>0.05$) different indicating that the immunisation did not cause the production of *Cryptosporidium*-specific antibodies. No *Cryptosporidium*-specific antibodies were detected in the serum of group W mice. The slight difference between the results for group W and group E mice prior to immunisation was most probably due to variation in the sensitivity of the instrument on different days.

Table 1. Comparison of the fluorescence intensity of *Cryptosporidium* oocysts stained with serum (diluted 1 in 1000) from group E and W mice and then stained with an anti-IgG or an anti-IgM fluorescently labelled antibody. Serum was tested prior to immunisation and then 21 days after the second immunisation.

	IgG specific antibody		IgM specific antibody	
mouse number	Pre-immunisation	Post-immunisation	Pre-immunisation	Post-immunisation
E1	12	1117	12	111
E2	9	460	11	121
E3	10	195	11	74
E4	10	1762	13	258
E5	12	550	12	33
W1	36	39	16	17
W2	45	32	27	14
W3	36	37	27	13
W4	42	30	25	27
W5	47	40	16	15
Positive* control (IgM)	42		978	
Negative Control	27		17	

ND - not determined

* a IgM monoclonal antibody specific to *Cryptosporidium* was used as a positive control. A IgG positive control was not available.

Hybridomas

Mouse number 4 from group E was sacrificed and the spleen cells fused with mouse myeloma cells. Screening of the resulting 230 clones identified six clones that were producing *Cryptosporidium*-specific antibody. Five of the clones (clones P9F1, P6G7, P6B11, P11D5 and P7D5) were found to be producing IgM antibody specific to the surface of *Cryptosporidium* oocysts. One clone (CRY104) was producing IgG1 antibody specific to the surface of *Cryptosporidium* oocysts.

Fusions were not attempted with mice from group W because of the poor immunological response to *Cryptosporidium* that was observed.

Evaluation of monoclonal antibodies

Results of flow cytometric analysis of water samples seeded with oocysts and stained with some of the *Cryptosporidium*-specific monoclonal antibodies are presented in Figure 1. Note the differences in the position of the population of oocysts along the Y axis. The oocyst population is closer to the top of the dotplot due for the sample stained with 8C12 (CRY104) than for any of the other samples. This is because the oocysts are fluorescing more brightly in this sample than any other sample. The fluorescence of the debris particles (below the ocysts) is not brighter in the 8C12 (CRY104) sample than in the unstained control. The separation between the oocysts and the debris particles is greatest in the 8C12 (CRY104) stained sample. This suggests that this antibody is most useful for staining *Cryptosporidium* oocysts in water samples. In comparision, the sample stained with 6G7 shows in increase in the fluorescence of some the debris particles (to the right of the oocysts) when compared to the unstained control. This is due to this antibody binding to some of the debris particles and suggests that this antibody may not be useful for staining *Cryptosporidium* oocysts in water samples.

CONCLUSIONS

A strong immunological response to the surface of *Cryptosporidium* oocysts was produced by immunising mice with a partially purified sample of the outer layer of the oocyst wall. Immunising mice with purified oocyst walls did not produce a strong immunological response. This would suggest that there is either immune suppression by a componant of the oocyst wall or that the natural presentation of the surface antigens on the oocyst walls does not produce an immunological response. The present inventors have

overcome this lack of an immunological response to the surface of *Cryptosporidium* oocysts by partially purifying the surface antigen.

Fusion of spleen cells from one mouse immunised with the purified sample of the outer layer of the oocyst wall resulted in six (6) hybridomas that produce antibody that is specific to the surface of the oocyst wall. One of these six antibodies is of the IgG1 immunological subclass, the remaining five antibodies are of the IgM subclass. The IgG1 antibody appears to be superior to the IgM antibodies for staining *Cryptosporidium* oocysts in water samples.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this Nineteenth day of May 1997

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Figure 1. Comparison of antibodies for staining oocysts in water samples. Axis are side scatter (X axis) versus green fluorescence (Y axis). Note the differences in the separation between the oocysts and the debris particles.